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TITLE: Evaluation of the immunologic impact of RAF Inhibitors to Guide Optimal Combination of RAF inhibitors and Immunotherapy for the treatment of Advanced Melanoma

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| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT During this first year of funding, we have made the following important observations. (1) T cells activated in vitro in the presence of BRAF inhibitors demonstrate a pattern of paradoxical activation characterized by upregulation of activation markers (CD69, PD-1, ICOS) and hyperactivation of the ERK signaling pathway. (2) T cells activated in vivo in the presence of BRAF inhibitors also demonstrate a pattern of paradoxical activation demonstrated by increased T cell expansion in vivo and hyperactivation of the ERK signaling pathway. (3) T cells activated in vitro in the presence of MEK inhibitors demonstrate inhibited upregulation of activation markers (CD69, PD-1, ICOS) and inhibition of the ERK signaling pathway. (4) T cells activated in vivo in the presence of MEK inhibitors also demonstrate a pattern of diminished activation demonstrated by lower T cell expansion in vivo and inhibition of the ERK signaling pathway. These first two observations have been reported in a manuscript that has been accepted for publication in the Journal Cancer Immunology Research. In addition, we have completed the following milestones that will form the foundation for future work: (1) regulatory approval for mouse studies, (2) regulatory approval for human correlative studies and 3) expansion of the BRAF/PTEN mouse colony. | | | | | |
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INTRODUCTION:

Our hypothesis is that combination therapy with MAPK inhibitors and immunotherapy will result in more rapid and durable control of melanoma than either modality alone. This hypothesis has gained support from several recent publications.¹⁻⁴ Additionally, this grant has supported work that resulted in the following publication :

Callahan, et al. Paradoxical activation of T cells via augmented ERK signaling mediated by a RAF inhibitor. **Cancer Immunology Research**. 2(1): 70-9. 2014.

The work was supported by several sources, but the following figures were supported by the DOD Visionary Postdoctoral Grant, as noted below (the manuscript is attached as Appendix 1).

Figure 1A-D (Corresponding to Aim 1a)

Figure 2 (Corresponding to Aim 1a)

Figure 3 (Corresponding to Aim 1a)

Figure 4 (Corresponding to Aim 1b)

Supplementary Figure 1 (Corresponding to Aim 1a)

Supplementary Figure 3 (Corresponding to Aim 1a)

Supplementary Figure 4 (Corresponding to Aim 1b)

Supplementary Figure 5 (Corresponding to Aim 1b)

According to the Statement of Work we have focused on the following areas described below:

1a) Test the effect of targeted inhibitors on expression of clinically relevant markers (ICOS, CTLA-4, PD-1, ki67) in T cells activated in vitro.

This first aim has been explored and is described in the attached manuscript (Appendix 1). Specific observations that we report upon in this manuscript include a description of a pattern of paradoxical activation of T cells exposed to BRAF inhibitors that is reflected in the upregulation of activation markers and in T cell proliferation in vitro (measured by ki67 upregulation). This is seen in Jurkat cells (Appendix 1 Figure 1A) and in healthy donor CD4 and CD8 positive T cells (Appendix 1 Figure 1 C and D). This pattern of paradoxical activation is seen in T cells activated by anti-CD3 antibody and by T cells activated in an antigen specific fashion using peptide pulsed APCs (Appendix 1 Figure 2). The mechanism of paradoxical activation in T cells is best explained by increased signaling via phosphorylated ERK, as demonstrated in vitro (Appendix 1 Figure 3). The effect of BRAF inhibitor treatment is contrasted to the effect of the MEK inhibitor, which attenuates T cell activation (Appendix 1 Figure 3E). In fact, the paradoxical activation of T cells by the BRAF inhibitor may be reversed by the additional of a MEK inhibitor. Moreover, the addition of MEK inhibitor to T cell culture results in diminished upregulation of a host of activation markers including PD-1, CD25, ICOS, and CD69 (see Figure 1 below). Some markers appear to be more robustly inhibited (CD25 and PD-1) while other appear to be more modestly reduced (CD69, ICOS). Additional

studies expanding the repertoire of activation markers evaluated and comparing BRAF and MEK inhibitors are ongoing.

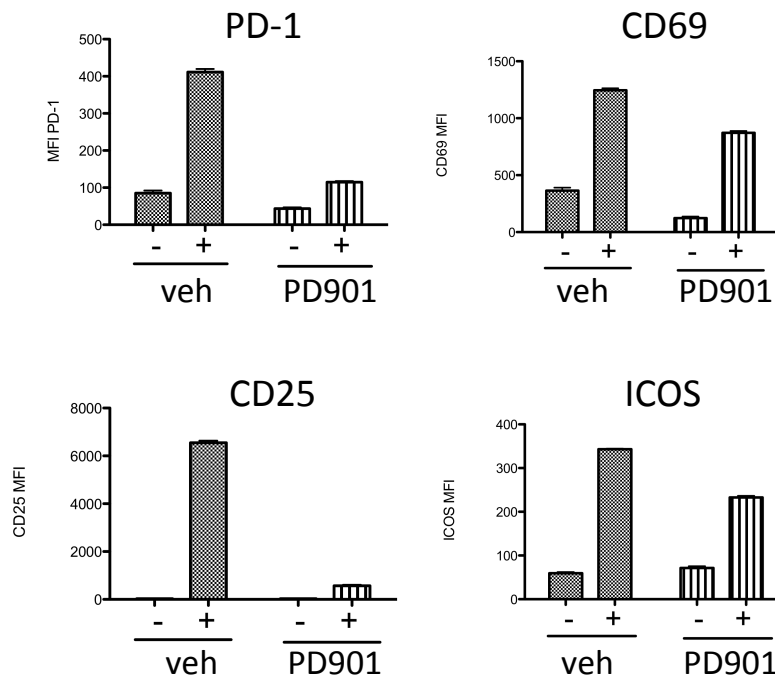


Figure 1. MEK inhibitors attenuate the upregulation of activation markers in T cells cultured in vitro. T cells cultured in the presence of vehicle control (veh) or MEK inhibitor PD325901 (PD901) were activated with anti-CD3 antibody. After twenty-four hours, expression of activation markers PD-1, CD69, CD25, and ICOS were measured by flow cytometry. Error bars represent SD for samples analyzed in triplicate.

Additionally, and outside of the published manuscript, we have evaluated a wider array of T cell activation markers and found that some markers follow this pattern of paradoxical activation, whereas other are simply inhibited in the presence of the RAFi XL281. In this analysis, we have evaluated the expression of the following, potentially clinically relevant T cell activation markers in vitro : ICOS, CTLA-4, CD69, PD-1, LAG-3 and ki67. As shown in Figure 2, we see two distinct patterns with some markers (ICOS, CTLA-4, and CD69) showing a clear pattern of paradoxical activation, as previously observed for CD69 in the published manuscript and prior preliminary data. Other marks, in contrast, show a pattern of inhibition in the presence of the RAFi XL281 (PD-1, LAG-3, ki67).

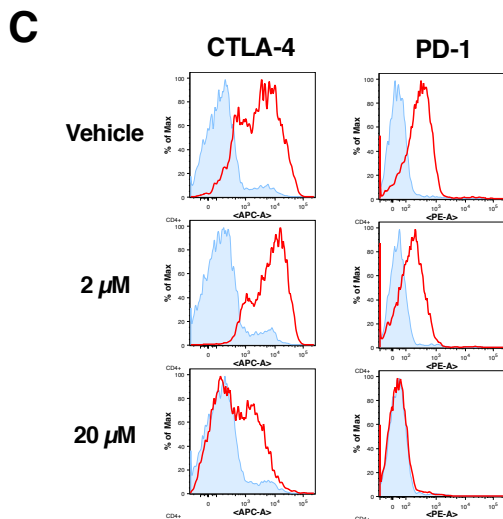
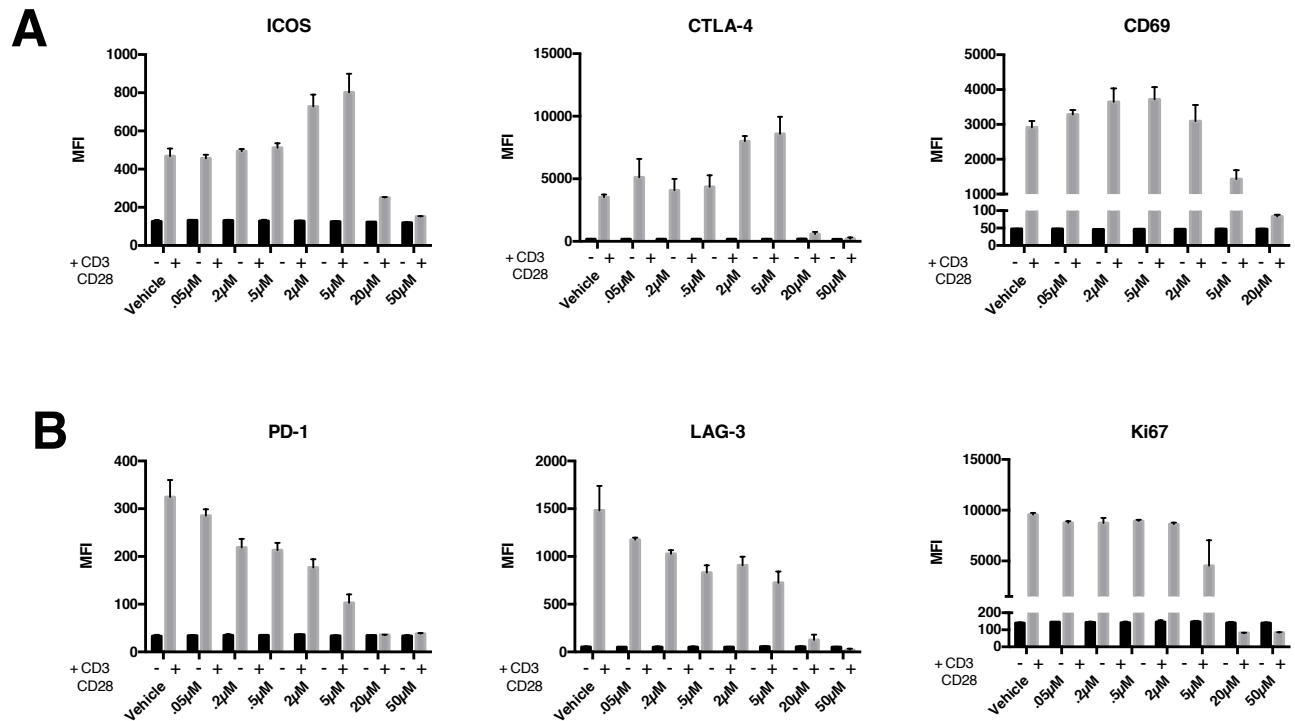


Figure 2: XL281 effects Murine CD4⁺ T cell activation in a concentration dependent manner.

Purified murine CD4⁺ T cells were activated with a combination of anti-CD3 and anti-CD28 antibody in the presence of titrated concentrations of XL281 ranging from .002µM to 20µM. Expression of activation markers was measured by flow cytometry after 24, 48, and 72 hours in culture and quantified using the MFI, median fluorescence intensity, for each activation marker. (A) XL281 enhances expression of activation markers ICOS, CTLA-4 and CD69 in a concentration dependent manner. Graphs depict ICOS (48 hours) CTLA-4 (72 hours) and CD69 (24 hours); black bars represent unstimulated conditions and gray bars represent stimulated conditions. Samples were treated and analyzed in triplicate and error bars represent standard error. Expression levels peak at a concentration of 2µM; at 20µM, the highest concentration tested, expression of these markers decrease below that of the cells treated with vehicle

(B) XL281 decreases expression of activation markers PD-1, LAG-3 and Ki67 in a concentration dependent manner. Graphs depict PD-1 (48 hours) LAG-3 (48 hours) and Ki67 (48 hours); black bars represent unstimulated conditions and gray bars represent stimulated conditions. Samples were treated and analyzed in triplicate and error bars represent standard error. Expression levels decrease with increasing doses of XL281 in a concentration dependent manner from the vehicle control. (C) Examples of histograms demonstrating staining for CTLA-4 (left) and PD-1 (right) for cells treated with vehicle, 2µM, or 20µM of XL281. red lines represent stimulated cells and blue lines represent unstimulated cells.

1b) Evaluate the effect of targeted inhibitors on activation and expansion of tumor-antigen specific transgenic T cells in vivo.

This aim has been explored and is described in the attached manuscript (Appendix 1). Specific observations that we report upon in this manuscript expand upon the initial observations on T cells activated in vitro by testing the impact of BRAF inhibitors on T cell activation in vivo. Specifically, we report that T cell expansion after antigen-specific stimulation is increased in a dose-dependent fashion in the presence of a BRAF inhibitor. (Appendix 1 Figure 4 A) Furthermore, paradoxical ERK pathway activation is tested ex vivo in mice treated systemically with BRAF inhibitor and we find that BRAF inhibitor increases ERK signaling, as previously described in vitro. (Appendix 1 Figure 4 B and C)

Additional experiments comparing the effects of BRAF and MEK inhibitors on T cell expansion in vivo suggest that (as seen in vitro), these two inhibitors that can have similar effects on tumor cells, have very different effects on T cells. Specifically, T cells stimulated in an antigen-specific fashion in vivo have robust expansion in the presence of BRAF inhibitor, but greatly diminished in the presence of MEK inhibitor (Figure 2, below).

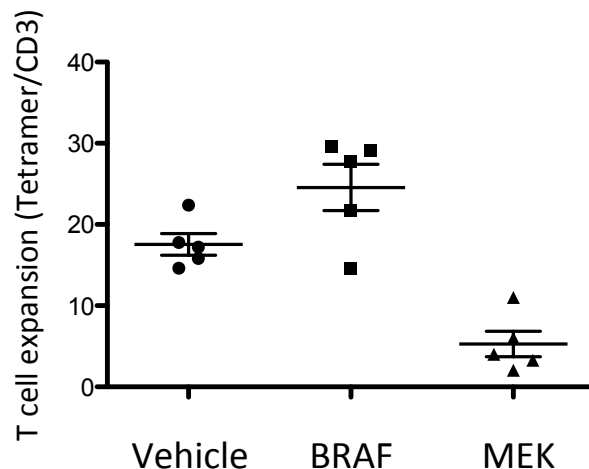


Figure 3. MEK inhibitors and BRAF inhibitors have opposing effects on T cells activated in vivo. Mice treated systemically with a vehicle control, the BRAF inhibitors PLX4720 or the MEK inhibitor PD325901 were immunized with peptide to expand antigen-specific TCR transgenic T cells. After 5 days, the expansion of antigen specific T cells was quantified by flow cytometry. Five mice were treated in each group and errors bars represent SD.

In addition, we are well underway in exploring the impact of MEKi on antigen-specific T cell activation in vitro/ex vivo. Using the experimental setup described in our published manuscript (Appendix 1, Supplementary Figure 4) and described below (Figure 4).

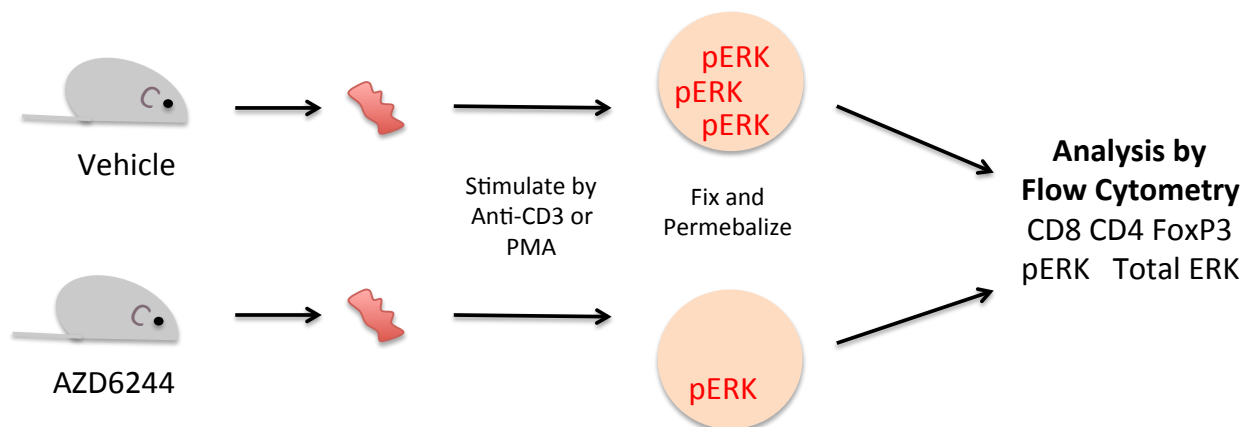


Figure 4. Mice treated systemically with the MEKi AZD6244 or vehicle control were treated for 4-5 days. Spleens were harvested and immediately ex vivo, splenocytes were stimulated and then fixed for staining with antibodies specific for pERK or ERK and T cell subset markers.

Using this approach, we have been able to explore some features of the effect that MEKi have on splenocyte activation ex vivo, as described below. We have found the MEKi appear to block activation of CD4+ (T eff), CD4+ FoxP3+ (T reg) and CD8+ T cell populations (Figure 5). Moreover, this effect is dose dependent (Figure 5). I also appears to be true for multiple MEKi (Figure 6, compare AZD6344 and PD901).

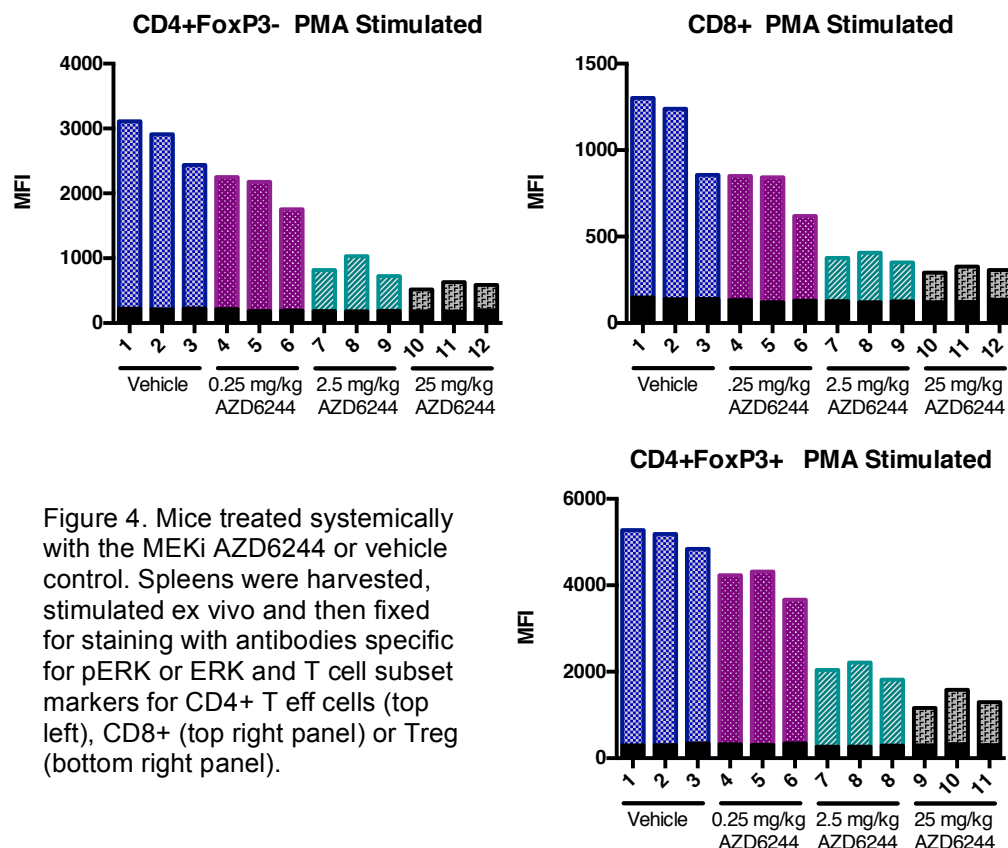


Figure 4. Mice treated systemically with the MEKi AZD6244 or vehicle control. Spleens were harvested, stimulated ex vivo and then fixed for staining with antibodies specific for pERK or ERK and T cell subset markers for CD4+ T eff cells (top left), CD8+ (top right panel) or Treg (bottom right panel).

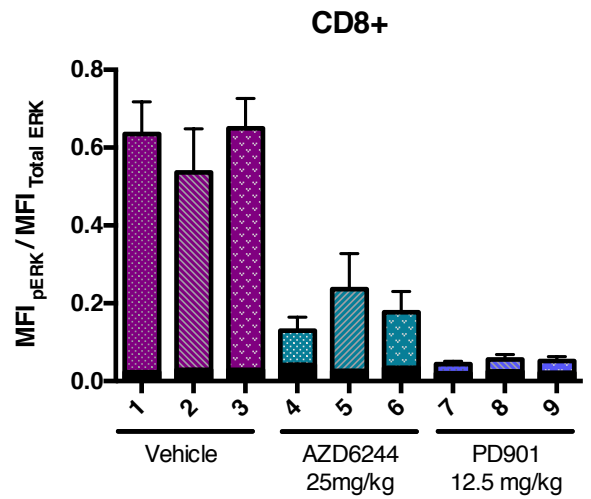
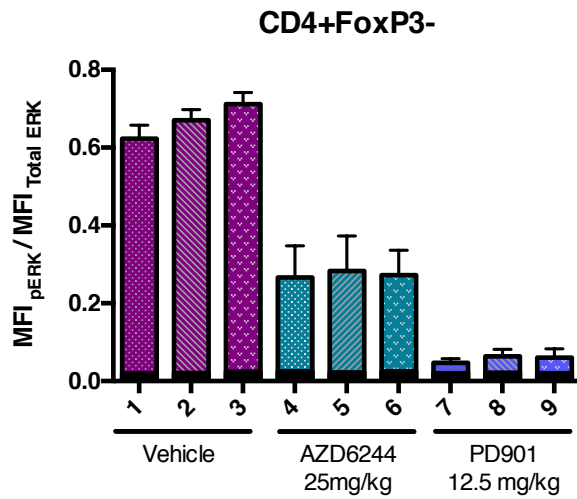
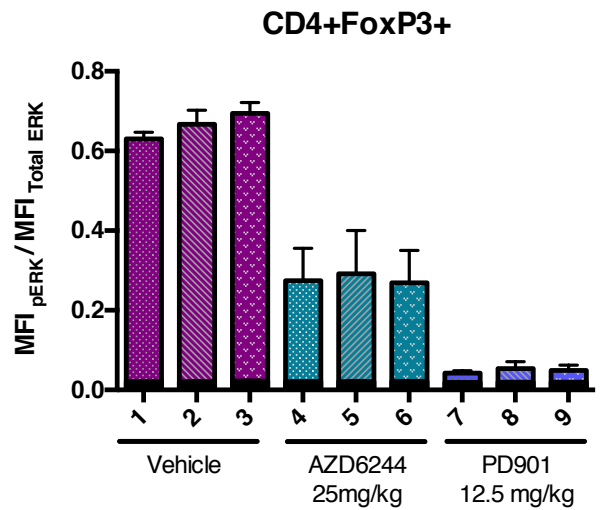


Figure 5. Mice treated systemically with the MEKi AZD6244 or PD901 or vehicle control. Spleens were harvested, stimulated ex vivo and then fixed for staining with antibodies specific for pERK or ERK and T cell subset markers for CD4+ T eff cells (top left), CD8+ (top right panel) or Treg (bottom right panel).



Extending these studies, we anticipate being able to better answer the question, when would the ideal time be to add checkpoint blocking antibodies and how might these combinations enhance or impair T cell activation ?

1d) Correlate pre-clinical findings by evaluating banked samples of T cells from patient previously treated with PLX4720 or AZD6244.

During this period of funding, we have obtained IRB approval for the analysis of these human samples and developed a flow cytometry panel of this analysis, as demonstrated in Figure 3, below. The development of an appropriate panel for multiparametric flow cytometry was a challenge that was overcome by expanding the testing of antibody

(2) Characterize the effect of targeted inhibitors on the anti-tumor activity of checkpoint

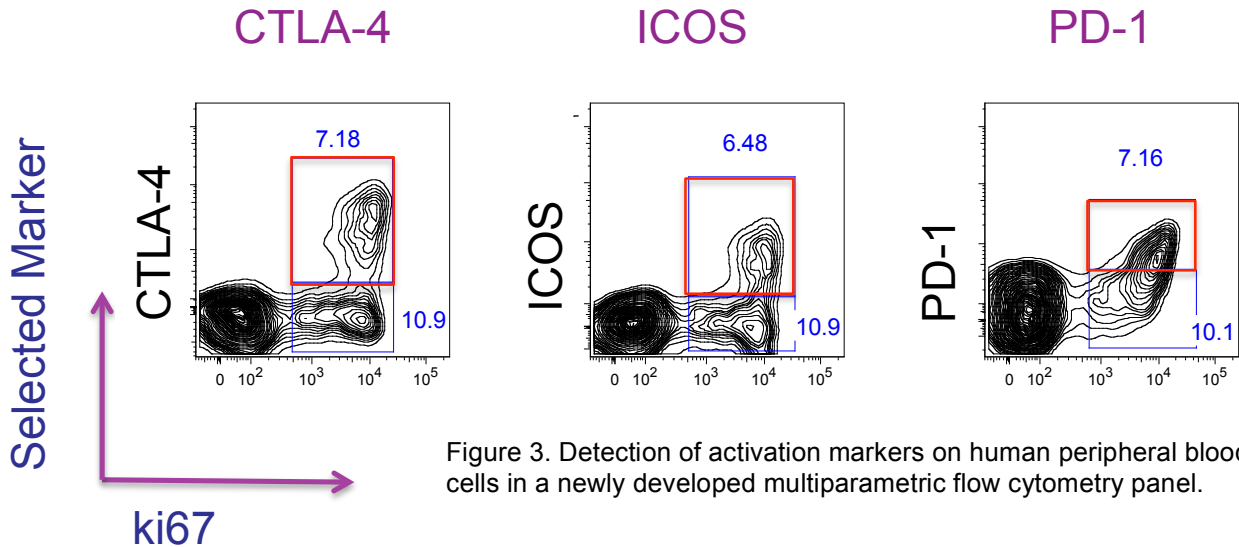


Figure 3. Detection of activation markers on human peripheral blood T cells in a newly developed multiparametric flow cytometry panel.

blocking antibodies (CTLA-4, PD-1) in an immunocompetant mouse model of BRAF mutant melanoma.

During this period of funding, we have obtained approval for the animal protocol and we have worked to expand the mouse colony of transgenic mice in order to support the experiments we have planned for Years 2-3.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by upregulation of T cell activation markers in vitro
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by upregulation of T cell activation markers in vitro
- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by enhanced proliferation of T cells (ki67, CFSE dilution) in vitro
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by proliferation of T cells (ki67, CFSE dilution) in vitro
- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by increased ERK signaling – supporting the mechanism of paradoxical activation
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by decreased ERK signaling .
- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by enhanced proliferation of T cell in vivo
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by proliferation of T cells in vivo.
- Development of a human T cell activation multiparametric flow cytometry panel
- Development and expansion of the mouse transgenic (BRAF/PTEN) colony to support mouse experiments in Years 2-3.

REPORTABLE OUTCOMES:

The following reportable outcomes have been accomplished during this funding period.

- Manuscripts published

Callahan, et al. Paradoxical activation of T cells via augmented ERK signaling mediated by a RAF inhibitor. **Cancer Immunology Research**. 2(1): 70-9. 2014.

- Abstracts and presentations

The following abstracts/presentation have been accepted during this funding period:

1. **Rational development of combinations of immunotherapies and targeted pathway inhibitors** AACR Annual Meeting, April 2013, Washington, DC
2. **Concentration Dependent Effects of RAF targeted therapies on human T cells** Cancer Immunotherapy Consortium, April 2013, Washington, DC
3. **Benefits and drawbacks of combinations of BRAF inhibitors and immunotherapy** Perspectives in Melanoma XVI, September 2013, Baltimore, MD

- Employment or research opportunities applied for and/or received based on experience/training supported by this award

Margaret Callahan, MD, PhD was appointed to a faculty position (Assistant Attending) at the Memorial Sloan-Kettering Cancer Center as a result of experience/training supported by this award.

CONCLUSION:

These studies characterizing the paradoxical T cell activation by BRAF inhibitors that result in increased T cells upregulation of activation markers, cytokines and proliferation in vitro and in vivo has several implications for future research and for the clinical development of these combination therapies. These findings represent one mechanism that may be exploited to maximize the clinical benefit of combination therapies, or suggest one mechanism that may explain toxicities that have recently been reported for these combination therapies.⁵ Our finding thus far with BRAF inhibitors are contrasted to our observations with MEK inhibitor treatment where T cell activation (including upregulation of PD-1, ICOS, CD25, CD69) are diminished in the presence of drug. These findings suggest a testable model where BRAF inhibitors are likely to combine with immunotherapies to generate robust, long-lasting anti-tumor T cell responses whereas MEK inhibitors may compromise the generation of long-lasting T cell memory: a hypothesis that will be tested in the experiments in mouse models planned for year 2-3. The presently designed experiments will be modified (added to) to specifically better characterize the generation of long-lasting anti-tumor immune responses. Specifically, we will plan to do a tumor re-challenge in mice who initially reject tumor after treatment with the combination of immunotherapy to additionally test the establishment of long-term T cell memory against tumor.

These studies have several implications for the development of combination therapies in the clinic and have generated new questions to be explored at the bench and in the clinic including:

1. Can paradoxical activation be exploited to enhance the anti-tumor T cell activity of immunotherapies ?
2. Can paradoxical activation of T cells be exploited in other clinical scenarios (i.e. vaccines) where robust T cell activation is desired ?
3. Will paradoxical activation be a liability for combination therapy and how would this impact the toxicity profile for combination therapies ?
4. How will the T cell effects of MEK inhibitors effect the clinical activity of combination therapy in the short term ? in the long term (T cell memory) ?
5. Will triple combinations (BRAF, MEK, and checkpoint blockade) be superior or inferior to double combinations (BRAF and checkpoint blockade) ?

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